Detection of *Rickettsia tsutsugamushi* by Gene Amplification Using Polymerase Chain Reaction Techniques[a]

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INTRODUCTION

Scrub typhus remains a major cause of febrile illness throughout the Asia-Pacific region.[1-3] Delay in diagnosis or the absence of a differential diagnosis has been shown to result in significant mortality.[4,5] The specific diagnosis of this disease is generally based on retrospective serodiagnosis, because clinically useful, rapid methods for cultivation and identification of the etiologic agent, *Rickettsia tsutsugamushi*, are not available. The recent introduction of gene amplification by the polymerase chain reaction (PCR) has proven useful in the diagnosis of certain infectious diseases for which the agents are difficult to grow or detect.[6-8] The laboratory mouse provides a well-established animal model for the study of scrub typhus.[9,10] Using the murine scrub typhus model and DNA primers derived from the DNA sequence of the 56-kilodalton (kDa) antigen of the Karp strain of *R. tsutsugamushi*,[11,12] we have developed a rapid direct-agent detection system for *R. tsutsugamushi* which is based on PCR amplification of rickettsial DNA. We present here the first application of PCR methodology used for the detection of the etiologic agent of scrub typhus and show that its sensitivity and specificity are adequate for the detection of the sparse numbers of rickettsiae present during infection.

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MATERIALS AND METHODS

Test Specimen Preparation

Outbred mice (Crl: −1(ICR)BR, Charles River Laboratories, Inc., Wilmington, MA), were infected by single intraperitoneal (i.p.) inoculation of 0.2 ml of rickettsial suspension containing *1000 50% i.p. mouse lethal doses* (MLD_{50}) of the Karp strain *R. tsutsugamushi*.[10] Mice were sacrificed and bled on day 9 post-inoculation (p.i.), when titers had previously been established to be $3–5 \times 10^3$ MLD_{50}/ml.[10] To establish detection limits, samples of infected mouse blood were diluted (10^{-1} to 10^{-4}) with uninfected mouse blood prior to DNA extraction. Upon collection, all mouse blood was stored at −70°C.

DNA Extraction

Genomic DNA was prepared as previously described[12,13] from purified suspensions of *R. tsutsugamushi*, strains Karp, Gilliam, and Kato; *R. typhi* Wilmington; *R. canada* McKiel; *R. rickettsii* R (ATCC VR-891); *Ehrlichia risticii* Illinois (ATCC VR-986); *Wolbachia persica* (ATCC VR-331); *Rochalimea quintana* Fuller; and *Escherichia coli* K12. DNA was extracted from frozen 6-ml aliquots of homogenized blood clot from infected and uninfected mice. These specimens were incubated with lysozyme at 20 mg/ml in Tris-HCl buffer for 45 min on ice. Following digestion with proteinase K at 1.0 mg/ml in 0.5% SDS, 50 m*M* Tris-HCl (pH 7.5), 0.4 *M* EDTA, for 1 h at 50°C, the DNA was purified by repeated phenol-chloroform extraction followed by ethanol precipitation. DNA pellets were resuspended in TE buffer (50 m*M* Tris-HCl, pH 7.5; 1 m*M* EDTA) and their concentrations measured by absorbance at 260 *nm*.

PCR Assay

A 1477–base pair region of the gene coding for the 56-kDa antigen of Karp strain *R. tsutsugamushi* DNA was amplified by PCR through either 35 cycles alone or with an additional 35 cycles according to the manufacturer's specifications using 12 μl each of two specific 20-mer oligonucleotide primers (TSU56R1, TSU56F1: sequence unpublished; Synthecell, Rockville, MD) and 0.5 μl of *Taq* polymerase (Geneamp DNA Amplification Reagent Kit, Perkin-Elmer Cetus, Norwalk, CT) in a total 100-μl reaction volume.[14] Primers were selected from GC-rich regions located near the 5′ and 3′ termini of the 1596–base pair gene. Each cycle started with a 30-s denaturation period at 94°C followed by a 2-min annealing step at 57°C and a 2-min extension step at 70°C using a DNA Thermal Cycler (Perkin-Elmer Cetus). Ten microliters of the amplified DNA was used in the subsequent, otherwise identical, 35-cycle amplification step. Amplification of 1-ng samples of extracted DNA was determined by visual detection of the expected ethidium bromide–stained bands following agarose gel electrophoresis of 5 μl of the PCR product in a 14-well minigel or 12 μl in an 8-well minigel. Amplified, electrophoresed DNA was blotted to nitrocellulose and hybridized to a specific 1477–base pair non-radioactively labeled, PCR-generated probe according to the specifications of the manufacturer (Genius DNA Labeling & Detection Kit, Boehringer Mannheim GMBH, Mannheim, West Germany).

RESULTS

In order to determine the specificity of the two selected 20-mer *R. tsutsugamushi* primers, the PCR-amplified DNA products of eight rickettsial procaryotes were examined by direct visualization of ethidium bromide–stained gels (FIG. 1.).

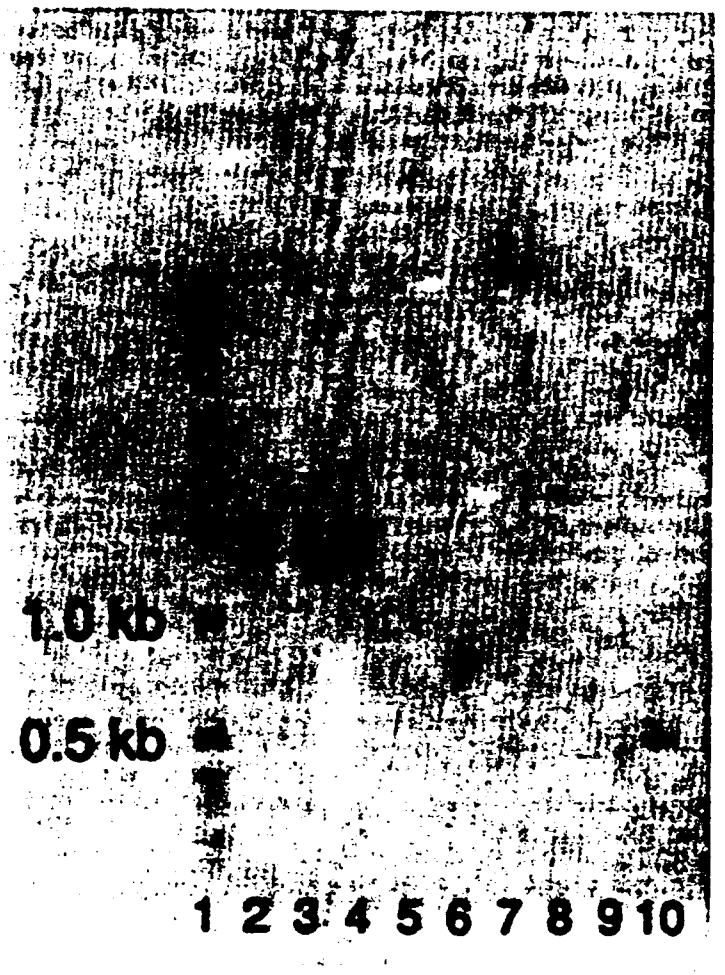

FIGURE 1. Agarose gel electrophoresis of PCR-amplified DNA from various Rickettsiales. **(Lane 1)** 1-kilobase (kb) DNA marker ladder. **(Lanes 2–4)** *R. tsutsugamushi*, strains **(lane 2)** Karp, **(lane 3)** Kato, **(lane 4)** Gilliam; **(lane 5)** *Ehrlichia risticii* Illinois (ATCC VR-986); **(lane 6)** *Rochalimea quintana* Fuller; **(lane 7)** *Wolbachia persica* (ATCC VR-331); **(lane 8)** *R. canada* McKiel; **(lane 9)** *R. typhi* Wilmington; **(lane 10)** *R. rickettsii* R (ATCC VR-891).

Single bands corresponding to amplified Karp, Gilliam, and Kato 56-kDa *R. tsutsugamushi* DNA were observed with the relative migration pattern predicted for the amplified DNA, whereas no bands of similar size were seen for the other species. Weakly staining bands corresponding to 0.8 kb (*Rochalimea quintana*) and 0.5 kb (*R. rickettsii*) were also observed. The PCR of *Escherichia coli* (K12) DNA failed to show any detectable band (data not shown).

DNA was extracted from each of a series of serially diluted *R. tsutsugamushi*–infected mouse blood specimens, the samples were PCR amplified for 35 cycles, and the products were examined for the presence of the expected ethidium bromide–stained band (FIG. 2). Specific bands were detected for blood dilutions of up to 1 : 100. When portions of the 1 : 100 and 1 : 1000 dilutions of amplified DNA were subjected to an additional 35 cycles of PCR, the bands were again detectable at a dilution of 1 : 100 but still were not detectable at greater dilution (data not shown).

To determine if detection sensitivity could be further enhanced, 35- and 70-cycle-amplified DNA from each dilution of infected mouse blood was subjected to Southern hybridization using amplified non-radioactive digoxigenin-11-dUTP–labeled DNA as probe. Specific hybridization of the 35-cycle-amplified DNA was detectable out to a dilution of 1 : 1000 of the original infected mouse blood (FIG. 3). There was no evident non-specific hybridization with the 1-kb DNA marker ladder. Hybridization of the 1 : 100 and 1 : 1000 dilutions of DNA which were amplified an additional 35 cycles showed specific hybridization at the 1 : 100 dilution only, thus failing to show any greater sensitivity than that seen with ethidium bromide staining (data not shown).

DISCUSSION

In order to establish PCR methodology and the suitability of selected primers as tools in the diagnosis of scrub typhus, it was necessary to establish the sensitivity and specificity of the test. Strain diversity within the species *R. tsutsugamushi* is well established.[15–19] In fact, antigenic analysis by direct immunofluorescence has shown that concomitant infection with multiple *R. tsutsugamushi* serotypes can occur in humans,[15] in small feral mammals,[17] and within vector chiggers[18,19] in highly endemic areas. Thus it was essential to show that the pair of primers selected for the PCR was genetically conserved and could be used to amplify multiple strains of *R. tsutsugamushi*. On the other hand, it was also essential that the conserved primers not be present in other bacterial species. As shown in FIGURE 1, ethidium bromide–stained bands are clearly evident for the three most commonly studied strains, i.e., Karp, Gilliam and Kato, though the Karp strain alone was used in the sequence determinations of the 56-kDa region. This finding indicates that at least the regions corresponding to the 20–base pair primers are conserved within the three *R. tsutsugamushi* strains examined. The presence of a group-reactive region found within the 56-kDa antigen is also supported by the earlier finding that cross-reactive antigens of similar molecular weights are present in Kato and Gilliam strains, as demonstrated by reaction with affinity-purified antibody.[12,16] Clearly, an examination of strains isolated from other febrile cases of scrub typhus will be necessary to show the intragroup sensitivity of the primers. Specificity of the system is further shown by the absence, in these stained gels, of similar-sized bands within any of the other rickettsial species examined.

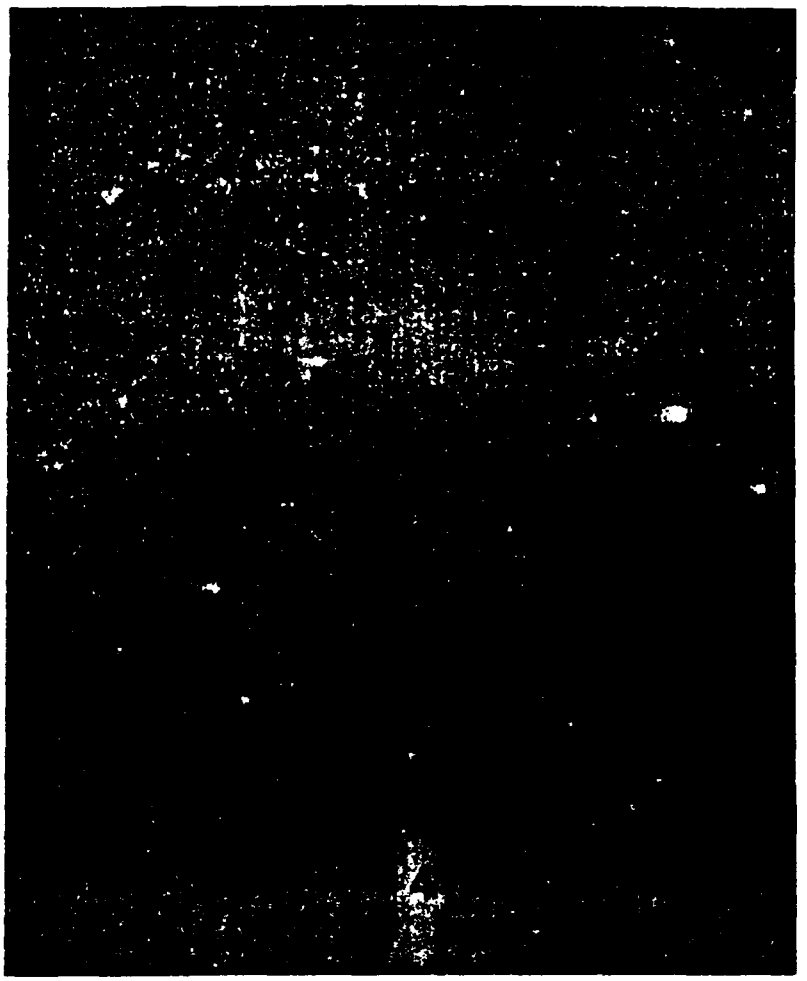

FIGURE 2. Agarose gel electrophoresis of PCR-amplified DNA extracted from 6-ml samples of serially diluted Karp strain *R. tsutsugamushi*–infected mouse blood. Each lane represents amplified DNA from a 35-cycle PCR assay of DNA samples. **(Lane 1)** 1-kb DNA marker ladder. **(Lane 2)** Undiluted infected mouse blood ($3–5 \times 10^4$ MLD_{50}), **(lane 3)** 1 : 10 dilution of infected mouse blood ($3–5 \times 10^3$ MLD_{50}), **(lane 4)** 1 : 100 dilution of infected mouse blood ($3–5 \times 10^2$ MLD_{50}), **(lane 5)** 1 : 1000 dilution of infected mouse blood ($3–5 \times 10^1$ MLD_{50}), **(lane 6)** empty lane, **(lane 7)** uninfected mouse blood control, **(lane 8)** empty lane.

We believe that separate extractions of DNA from infected mouse blood diluted with uninfected blood, rather than dilutions of the extracted DNA alone, more accurately reflect the diseased state. In low-level rickettsemias, the greater proportion of the extracted "contaminating" eucaryotic DNA in comparison to the small proportion of rickettsial DNA could potentially interfere with attempts to detect the agent DNA. A sensitivity determination based on PCR amplification

of dilutions of the extracted DNA would not have the excesses of the eucaryotic DNA to interfere in the reaction. PCR amplification of DNA extracted from the infected mouse blood showed specific bands at 1 : 1000 dilution, corresponding to detection of 3–5 MLD_{50}/ml of infected blood.

Although there is relatively little published quantitative data on the levels of rickettsemia during the various rickettsial diseases, Shirai *et al.*,[20] using mouse endpoint titration, reported detecting from 9 to 160 MLD_{50} of *R. tsutsugamushi* per milliliter of human blood between days 2 and 14 of clinical scrub typhus. If these blood levels of rickettsiae are correct, an assay sensitivity of as few as 5 MLD_{50}/ml would be necessary to permit a sensitive and accurate diagnosis of scrub typhus soon after the onset of symptoms. Our system shows such sensitivity.

The suitability of the PCR test for human disease is being evaluated on clinical materials collected from febrile patients from Southeast Asia who were diagnosed

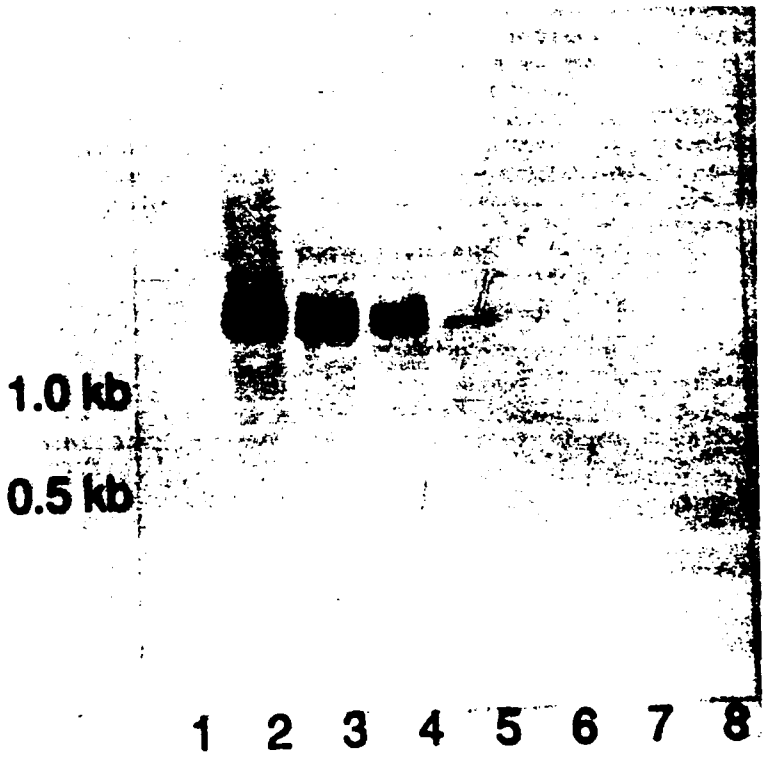

FIGURE 3. Southern blotting analysis of PCR-amplified product of DNA extracted from serial dilutions of blood of mice infected with Karp strain *R. tsutsugamushi* (blot is of gel from FIG. 2). **(Lane 1)** 1-kb DNA marker ladder. **(Lanes 2–5)** Infected mouse blood diluted **(lane 2)** 1 : 1, **(lane 3)** 1 : 10, **(lane 4)** 1 : 100, **(lane 5)** 1 : 1000, **(lane 6)** empty lane, **(lane 7)** uninfected mouse blood control, **(lane 8)** empty lane.

as having scrub typhus by clinical presentation and seroconversion. Thus, for the first time, the PCR may provide clinicians within the endemic regions a rapid, highly accurate agent detection system for the diagnosis of scrub typhus which can be used early in the course of illness when therapeutic intervention can be beneficial.

SUMMARY

Scrub typhus is commonly undiagnosed in endemic areas due, in part, to dependence on retrospective serodiagnosis. Since the etiologic agent, *R. tsutsugamushi*, will not grow in cell-free systems, a rapid direct-agent detection system such as provided by polymerase chain reaction (PCR) methodology is needed. Genes coding for the variable 56-kDa antigen of *R. tsutsugamushi* were amplified through 35 cycles using 20-mer oligonucleotide primers and *Taq* polymerase. Amplification of 1-ng samples of DNA extracted from purified prototype *R. tsutsugamushi* Karp, Gilliam, and Kato strains was detected by direct visual inspection of the electrophoresed, ethidium bromide–stained, specific bands. Specificity of the PCR was shown when PCR amplification of various non–scrub typhus rickettsial DNAs was unsuccessful. *R. tsutsugamushi* DNA extracted from the blood of infected mice could be PCR amplified and the 1477–base pair product detected by either direct visualization or by specific hybridization with amplified non-radioactive digoxigenin-11-dUTP–labeled Karp 56-kDa DNA probe.

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